REMARKS

Claims 1-62, 65-66 and 68-74 were previously pending in this application. By this amendment, Applicant is canceling claims 63-64, 67 and 73-74 without prejudice or disclaimer. Claims 1-59, 65 and 73-74 have been withdrawn. As a result claims 60-62, 66, and 68-72 are pending for examination with claims 60 and 66 being independent claims. New claims 75 has been added. No new matter has been added.

Rejections Under 35 U.S.C. §112

The Examiner has rejected claims 66 and 68-72 under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

1. The state of the prior art and the predictability or unpredictability in the art

The Examiner has rejected the claims on the basis that the ability to selectively target uncoupling or lysosomotrophic agents to appropriate target cells is unpredictable. The Examiner cites Bouillard et al., Cone, and Szoka et al. as teaching that although the ability to alter pH of lysosomes is routinely accomplished in the art, and Branch, Crooke, Bioworld article, Palu, Agrawal, and Chirila for the teaching that the ability to predictably and selectively target uncoupling or lysosomotrophic agents to appropriate target cells whereby treatment effects are provided in an organism, is currently not a routine matter in the art.

Each of the teachings of the cited references must be examined in the context of the whole reference and the field. Applicant has addressed each reference below. However, it is also noted that each of these references deals with delivery of nucleic acids for therapeutic benefit. Applicants claims are directed to binding peptides and molecules.

Branch and Crooke provide general reviews of the use of antisense molecules and their applications. Crooke reports on variability in the cellular uptake of oligonucleotides, Crooke also reports that many well-controlled studies have been reported in which antisense activity was conclusively demonstrated (page 24-25).

The BioWorld Today article reporting on a clinical trial by ISIS pharmaceuticals for the treatment of Crohn's disease using antisense targeting ICAM-1 reported that the placebo treatment was more successful than antisense treatment. This article cites a two part study, in which the first part showed 29% remission in patients compared with 14% in placebo-treated, and the second part showed 13% remission in patients compared with 22% in placebo-treated. The article does not make it clear as to whether the inconsistent data is a result of experimental design or the actual drug itself. There are a number of variations involved in this particular clinical trial, including the difficulty involved in evaluating Crohn's disease because of periods of exacerbation (see page 2, second column), which may explain the discrepancy. One of ordinary skill in the art would recognize that one unsuccessful clinical trial would not be indicative of the use of antisense treatment.

Palu et al. state that there is no ideal vector or a common strategy valid for all applications and each vector system will have to be tailored to the specific disease, and possess functions that enable efficient transfer and targeted expression of the transgene (see pages 3-4, abridging paragraph). One of ordinary skill in the art would be able to identify vectors suitable for their needs.

Chirila et al. state that there are many reports indicating enhanced therapeutic effects of antisense oligonucleotides. They also report on a study by Wagner which demonstrated that the localization of an antisense oligonucleotide to the nucleus of a cell or cytoplasm was dependent on the delivery method used (see page 327).

Agrawal et al. state that the cellular uptake of negatively charged oligonucleotides is one of the important factors in determining the efficacy of antisense oligonucleotides. Agrawal et al. also states that each antisense oligonucleotide should be studied in its own context and cell line (see page 80). One of ordinary skill would view this as standard practice.

Additionally, Applicant attaches hereto a recent paper by LeBowitz et al (Exhibit 1) describing both the state of the art delivery method for targeting molecules to lysosomes as well as a new method that they developed. Although the reference points out difficulties with conventional lysosomal delivery in order to highlight the benefits of their own delivery system, the introduction describes therapeutic procedures using such conventional technology. The

reference supports applicants assertions in the specification that peptides and other molecules can be delivered to the lysosome.

Accordingly, withdrawal of this rejection is respectfully requested.

2. The Amount of Direction or Guidance Presented in the Specification and the Presence of Absence of Working Examples

The Examiner states that the specification fails to teach the treatment of an infectious disease comprising the administration of a lysosomal targeted binding peptide UCP inhibitor or a lysosomal targeted binding molecule UCP inhibitor in an organism. According to the Examiner the specification fails to provide any particular guidance which resolves the known unpredictability in the art associated with the *in vivo* delivery of UCP inhibitors and treatment effects provided by the administration of such inhibitors to an organism and which treatment methods are provided for infectious disease or conditions comprising the administration by any route of lysosomal targeted binding peptide UCP inhibitors or lysosomal targeted binding molecule UCP inhibitors.

The Applicant has provided a description of inhibiting lysosomal UCP activity to treat infections on page 48 of the specification. The inhibition of lysosomal UCP promotes the development of an acidic intra-lysosomal environment which promotes antigen presentation. When antigen presenting cells are presented with antigen in this manner the antigen can be processed and presented on the cell surface, allowing an effective antigen specific immune response against the antigen (page 48, lines 11-23). A second method for treating infectious disease using inhibitors of lysosomal UCP promotes respiratory burst which is useful for treating intracellular pathogens. The Applicant provides details of this method on page 48, lines 24-31 of the specification. Applicants have not seen any evidence that administration of lysosomal UCP inhibitors is unpredictable. The references cited above relate to the delivery of nucleic acids. Although the references describes difficulties associated with these technologies, it does not render administration of lysosomal UCP inhibitors unpredictable.

Accordingly, withdrawal of this rejection is respectfully requested.

3. Breadth of the Claims and Quantity of Experimentation Required

The Examiner states that the breadth of the claims is broad and that it would require undue experimentation to practice the invention. According to the Examiner, the findings that UCP2 knockout mice have increased resistance to Toxoplasma gondii, and that UCP inhibitors were found to regulate lysosomal pH in vitro, are not representative of the ability to treat infectious diseases in an organism comprising the administration of any lysosomal UCP inhibitor.

The claims are broad but not unpredictable. Applicant was the first to discover the presence of UCP in the lysosome and that the activity of UCP in the lysosome could be inhibited in order to treat infection. Applicant described a class of compounds that accomplish the inhibition and have taught how to administer the compounds to achieve the desired result. There is no requirement that applicant present actual in vivo data to demonstrate this finding. Applicant has presented the in vivo knockout data in support of the claims. Applicant should be entitled to broad claims based on the critical discovery relating to UCP in the lysosome. There is no evidence of record that the method is unpredictable.

Maintained Rejections

The Examiner has maintained the rejection of claims 60-62, 66 and 68-72 under U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s) had possession of the claimed invention. The Examiner refers to the reasons as set forth in the Office Action mailed January 14, 2003. The rejection is based on the genus of claimed compounds. The Examiner has requested that applicant point out the common structural features of the species within the genus.

The genus of compounds encompassed by independent claims 60 and 66 includes lysosomal targeted binding peptide and molecule. The species within this genus all share the common element that they bind to lysosomal UCP and thus interfere with the activity of lysosomal UCP. Each member of the genus has this property in common. It is believed that this common element should be enough to overcome the rejection. Applicant has, however, added a new claim that indicates that the binding peptide is an antibody. This narrow species claim should be free of the rejection in any case.

Accordingly, withdrawal of this rejection is respectfully requested.

CONCLUSION

In view of the foregoing amendments and remarks, this application should now be in condition for allowance. A notice to this effect is respectfully requested. If the Examiner believes, after this amendment, that the application is not in condition for allowance, the Examiner is requested to call the Applicant's attorney at the telephone number listed below.

If this response is not considered timely filed and if a request for an extension of time is otherwise absent, Applicant hereby requests any necessary extension of time. If there is a fee occasioned by this response, including an extension fee, that is not covered by an enclosed check, please charge any deficiency to Deposit Account No. 23/2825.

Respectfully submitted,
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Exhibit 1

Glycosylation-independent targeting enhances enzyme delivery to lysosomes and decreases storage in mucopolysaccharidosis type VII mice

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Contributed by William S. Sly, December 30, 2003

Enzyme-replacement therapy is an established means of treating lysosomal storage diseases. Infused therapeutic enzymes are targeted to lysosomes of affected cells by interactions with cell-surface receptors that recognize carbohydrate moieties, such as mannose and mannose 6-phosphate, on the enzymes. We have tested an alternative, peptide-based targeting system for delivery of enzymes to lysosomes in a murine mucopolysaccharidosis type VII (MPS VII) model. This strategy depends on the interaction of a fragment of insulin-like growth factor II (IGF-II), with the IGF-II binding site on the bifunctional, IGF-II cation-independent mannose 6-phosphate receptor. A chimeric protein containing a portion of mature human IGF-II fused to the C terminus of human β-glucuronidase was taken up by MPS VII fibroblasts in a mannose 6-phosphate-independent manner, and its uptake was inhibited by the addition of IGF-II. Furthermore, the tagged enzyme was delivered effectively to clinically significant tissues in MPS VII mice and was effective in reversing the storage pathology. The tagged enzyme was able to reduce storage in glomerular podocytes and osteoblasts at a dose at which untagged enzyme was much less effective. This peptide-based, glycosylation-independent lysosomal targeting system may enhance enzyme-replacement therapy for certain human lysosomal storage diseases.

β-glucuronidase | IGF-II/Man6-P receptor | receptor-mediated endocytosis | enzyme-replacement therapy | lysosomal storage disease

Lysosomal storage diseases (LSDs) are a class of >40 rare genetic disorders, each of which is caused by a deficiency in a specific lysosomal enzyme. As a consequence of the progressive accumulation of unmetabolized macromolecules in the lysosomes of cells in various tissues, the disease manifestations worsen over time (1). Individuals afflicted with an LSD can suffer from mild to severe physical and/or neurological abnormalities or can die at an early age. A therapeutic paradigm for the treatment of LSDs was established with the success of enzyme-replacement therapy (ERT) for the treatment of Gaucher disease (2, 3). The success of this therapeutic strategy relies on targeting the enzyme to specific cell-surface receptors and enzyme transport to the lysosome by receptor-mediated endocytosis after the missing enzyme is infused into the patient's bloodstream (reviewed in ref. 4).

In the case of Gaucher disease, the glucocerebroside storage product accumulates primarily in resident tissue macrophages, such as Kupffer cells, in the liver. Delivery of glucocerebrosidase to these cells was achieved by modifying the N-linked carbohydrate on the enzyme to expose core mannose residues (5, 6), enabling the enzyme to bind to the mannose receptor, which is highly abundant on cells of the reticuloendothelial system (7, 8).

In contrast to Gaucher disease, most of the other LSDs exhibit storage that involves cell types lacking the mannose receptor. Delivery of enzymes to these cells has taken advantage of another receptor, the IGF-II/cation-independent mannose 6-phosphate receptor (IGF-II/CI-MPR), which recognizes mannose 6-phosphate (Man6-P) moieties that are added to oligosaccharides on newly synthesized lysosomal enzymes in mammalian cells (9). This receptor–ligand interaction is a key element of the normal intracellular traffic pathway that delivers newly synthesized enzymes to the lysosome (10). The IGF-II/CI-MPR is present also on the surface of many mammalian cell types, enabling administered Man6-P-containing proteins to be targeted to cells in a wide variety of tissues (11).

For example, β-glucuronidase (GUS), α-galactosidase A, and α-L-iduronidase, the enzymes defective in mucopolysaccharidosis type VII (MPS VII), Fabry disease, and mucopolysaccharidosis type I (MPS I), respectively, are taken up into patient fibroblasts by binding to the IGF-II/CI-MPR, as demonstrated by the potency of free Man6-P as a competitive inhibitor of uptake (12–15). Each of these enzymes is effective in reversing lysosomal storage in many tissues in animal disease models. The requirement of Man6-P on enzymes for delivery to non-reticuloendothelial system (RES) cells has been demonstrated directly in the murine model of MPS VII (11). Recombinant α-galactosidase A (16) and α-L-iduronidase (14) are now approved for treatment of Fabry disease and MPS I in humans.

Production of effective Man6-P-targeted ERTs for some disorders is difficult for the following reasons. (*i*) Recombinant proteins tagged with Man6-P must be produced in mammalian systems, excluding the use of alternative expression systems such as bacteria, yeast, or insect cells, which do not produce the Man6-P modification; (*ii*) some lysosomal enzymes are poorly modified with Man6-P, even when expressed in mammalian cell culture systems, making it difficult to achieve effective targeting (17); and (*iii*) many lysosomal enzymes have a short half-life when injected into the bloodstream because of rapid clearance in the liver by other carbohydrate-recognizing receptors, particularly the mannose receptor that is highly abundant on Kupffer cells. For example, human placental GUS injected into rodents is cleared rapidly from the circulation by mannose receptors on reticuloendothelial cells (8).

A peptide-based targeting system for lysosomal enzymes might solve these problems by providing a means of glycosylation-independent lysosomal targeting (GILT). A peptide-based targeting system might be compatible with alternate expression systems, overcome problems associated with poor Man6-P phosphorylation, and be compatible with strategies that sought to

Abbreviations: GUS, β-glucuronidase; mGUS, murine GUS; hGUS, human GUS; IGF-II, insulin-like growth factor; IGF-II/CI-MPR, IGF-II/cation-independent mannose 6-phosphate receptor; Man6-P, mannose 6-phosphate; CI-MPR, cation-independent Man6-P receptor; LSD, lysosomal storage disease; MPS VII, mucopolysaccharidosis type VII; ERT, enzyme-replacement therapy; GILT, glycosylation-independent lysosomal targeting.

†W.S.S., the contributing member, has received a grant from Symbiontics, Inc., that supported this research. J.H.L. is the Chief Scientific Officer of Symbiontics, Inc., which provided financial support for this work. Symbiontics, Inc., has filed patents for the technology using the GILT tag on lysosomal enzymes for ERT.

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Table 1. Oligonucleotides used in the construction of the GILT cassette

Name	Sequence	Position
GILT 1	GCGGCGGCGAGCTGGTGGACACGCTGCAGTTCGTGTGCGGCGACCGCGGC	48–97 (top)
GILT 2	TTCTACTTCAGCCGCCCGGCCAGCCGCGTGAGCCGCCGCAGCCGCGGCAT	98–147 (top)
GILT 3	CGTGGAGGAGTGCTGCTTCCGCAGCTGCGACCTGGCGCTGCTGGAGACGT	148–197 (top)
GILT 4	ACTGCGCGACGCCGGCGAAGTCGGAGTAAGATCTAGAGCG	198–237 (top)
GILT 5	AGCGTGTCCACCAGCTCGCCGCCGCACAGCGTCTCGCTCGGGCGGTACGC	72–23 (bottom)
GILT 6	GGCTGGCCGGGCGGCTGAAGTAGAAGCCGCGGTCGCCGCACACGAACTGC	122–73 (bottom)
GILT 7	GCTGCGGAAGCAGCACTCCTCCACGATGCCGCGGCTGCGGCGGCTCACGC	172–123 (bottom)
GILT 8	CTCCGACTTCGCCGGCGTCGCGCAGTACGTCTCCAGCAGCGCCAGGTCGCA	223–173 (bottom)
GILT 9	CCGTCTAGAGCTCGGCGCGCCGGCGTACCGCCCGAGCGAGACGCTGT	1–47 (top)
GILT 10	CGCTCTAGATCTTACTCCGACTTCG	237–202 (bottom)
GILT 11	CCGTCTAGAGCTCGGCGCGCCGCTGTGCGGCGGCGAGCTGGTGGAC	1–67, Δ23–43 (top)

The name, sequence, and relative position of the oligonucleotides encoding the GILT tag are listed.

circumvent rapid clearance by the mannose receptor. For example, treatment of placental GUS with periodate, which reacts with cis diols in the carbohydrate, increases the bloodstream half-life of the enzyme by ≥20-fold (8, 18, 19). If this or another enzyme linked to a peptide-targeting moiety were periodate-treated, its ability to target lysosomes might remain intact and escape rapid clearance by the mannose receptor.

To develop a GILT system, we made use of the ability of the peptide hormone IGF-II to bind to the IGF-II/CI-MPR with high affinity (20, 21). We hypothesized that a portion of IGF-II (hereafter referred to as the "GILT tag") retaining the ability to bind to the IGF-II/CI-MPR would serve as an effective targeting moiety when fused to lysosomal enzymes. Such a GILT-tagged protein would target the identical receptor targeted by Man6-P (albeit to a distinct binding site), thereby sharing the identical endocytic pathway for lysosomal targeting with Man6-P-containing proteins (22–27).

We tested the validity of a GILT tag as a lysosomal targeting agent for ERT in a murine model of MPS VII, an LSD caused by a deficiency of the lysosomal hydrolyase murine GUS (mGUS), which catalyzes a step in the degradation of glycosaminoglycans (28, 29). Although the number of human patients suffering from MPS VII is very small, well characterized animal models are available that have been studied extensively (29–31). MPS VII mice lack the lysosomal enzyme mGUS and display a disease progression that is similar to the disease progression observed in human patients lacking human GUS (hGUS). Multiple infusions of mGUS have been shown to be effective in ameliorating many of the symptoms and their underlying causes in MPS VII mice (32, 33). The efficacy of the infused enzyme depends on its delivery to cell-surface receptors in a range of tissues in which the storage products accumulate. Thus, demonstration that a potential therapeutic enzyme is delivered effectively to target tissues is a good predictor of the likely clinical effectiveness of the therapy (31). We report here that the GILT tag is effective at delivering the chimeric protein hGUS-GILT to a wide range of tissues and cell types that exceeds the range of cell types targeted by native hGUS at the same dose.

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hGUS-GILT Cassettes. An IGF-II cassette encoding residues 8–67 of mature human IGF-II was synthesized by ligation of a series of overlapping oligonucleotides. Amino acids 1–7 of IGF-II were deleted to reduce the affinity of the tag for the IGF-I receptor and IGF binding proteins while preserving its affinity for the IGF-II/CI-MPR (see *Discussion*). To make the 67-aa mature IGF-II cassette, oligonucleotides GILT 1–9 were annealed and ligated (see Table 1). To incorporate the Δ1–7 deletion, the wild-type cassette was PCR-amplified by using oligonucleotides GILT 11 and 10. The resultant cassette, referred to as the GILT cassette, contained a unique *Asc*I restriction site at the 5′ terminus, which encodes a 3-aa bridge, Gly-Ala-Pro. The GILT cassette was fused to a gene cassette encoding hGUS with a deletion of the region encoding the 18 C-terminal residues. This cassette was produced by amplifying the *hGUS* cDNA cassette with the following primers: 5′ECO, CACGAATTCGCCAC-CATGGCCCGGGGGTCGGCGGTTGCCT; and 3′ECO, CGCGAATTCTTACTCCGACTTCGCCGGCGTCGCG-CAGT. Primer 3′ECO contained an *Asc*I site and, consequently, the PCR product could be fused in frame to the GILT cassette, creating the final hGUS-GILT cassette.

Expression of Recombinant Proteins in Chinese Hamster Ovary Cells. hGUS-GILT was expressed in Chinese hamster ovary cells by using the mammalian expression vector pCXN, as described (34, 35). The pCXN vector containing the hGUS-GILT cassette inserted into the *Eco*RI site was electroporated into Chinese hamster ovary cells at 25 μF and 1,200 V in a 0.4-cm cuvette. Selection of colonies and amplification were carried out in growth medium (MEM supplemented with 15% FBS/1.2 mM glutamine/50 μg/ml proline/1 mM pyruvate) plus 400 μg/ml G418 for 10–14 d.

The highest-producing chinese hamster ovary cell line was grown to confluency in triple flasks (Nunc) and fed with low-serum medium (Waymouth's MB 752/1 medium, supplemented with 2% FBS/1.2 mM glutamine/1 mM pyruvate) to collect enzyme for purification. The flasks were refed at 24-h intervals. Media from several flasks were pooled, centrifuged at 5,000 × *g* for 20 min at 4°C to remove detached cells, and frozen at −20°C for later use. Untagged recombinant hGUS was produced in a similar fashion.

Purification of Recombinant Proteins. Affinity chromatography conditions were essentially as described in Islam *et al.* (36). Conditioned medium from cells overexpressing the GUS-GILT fusion protein was filtered through a 0.22-μ filter. Sodium chloride (crystalline) was added to a final concentration of 0.5 M, and sodium azide was added to a final concentration of 0.025%. The medium was applied to a 5-ml column of anti-hGUS-conjugated Affigel 10, preequilibrated with antibody–Sepharose wash buffer containing 10 mM Tris (pH 7.5), 10 mM potassium phosphate, 0.5 M NaCl, and 0.025% sodium azide at a rate of 25 ml/h at 4°C. The column was washed at 36 ml/h with 20-column volumes of antibody–Sepharose wash buffer. The column was eluted at 36 ml/h with 50 ml of 10 mM sodium phosphate (pH 5.0) and 3.5 M $MgCl_2$. Fractions (4 ml) were collected and assayed for GUS activity. Fractions containing the fusion protein were pooled, diluted with an equal volume of P6 buffer (25 mM Tris, pH 7.5/1 mM β-glycerol phosphate/0.15 mM NaCl/0.025% sodium azide), and desalted over a BioGel P6

column (preequilibrated with P6 buffer). Fractions containing GUS activity were pooled and assayed. The specific activity of the fusion protein was comparable with that of native hGUS purified in a similar fashion (i.e., 4.5–5.0 × 10^6 units/mg). The fusion protein was stored frozen at −80°C in P6 buffer. For removal of carbohydrates, hGUS-GILT was treated with endoglycosidase F1 (ProZyme, San Leandro, CA) and used according to the manufacturer's instructions.

Uptake Assays. GUS-deficient GM04668 fibroblasts (Coriell Cell Repositories, Camden, NJ) were incubated in 12-well plates at 37°C and 5% CO_2 for 3 h with 4,000 units (nmol/h·ml) of purified enzyme per ml of uptake media containing Dulbecco's MEM (low glucose; GIBCO/BRL), 4 mM L-glutamine (GIBCO/BRL), and 2% BSA (Sigma). Some wells also contained either 2 mM Man6-P (Calbiochem) or 2.86 mM IGF-II (Cell Sciences, Canton, MA) as inhibitors. Cells were washed four times in PBS and then lysed in buffer containing 10 mM Tris (pH 7.5), 100 mM NaCl, 5 mM EDTA, and 1% Nonidet P-40. GUS activity was determined as described (37). Units of GUS present in the cellular fraction were normalized to the lysate protein concentration, as determined by using the bicinchoninic acid (BCA) protein assay (Pierce).

Animal Experiments. In these studies, MPS VII/E540A^{tg} mice were used (31). These mice carry an hGUS transgene that encodes an inactive enzyme, which induces immunotolerance to the human protein. To determine the biodistribution of the three forms of the enzyme in adult mice, six animals for each enzyme (seven for hGUS-GILT) were injected in the lateral tail vein with a dose of 1 mg/kg body weight of hGUS, hGUS-GILT, or hGUS-GILT-F1 in a volume of 125 μl of PBS solution. Additionally, six control animals received PBS buffer only. The animals were killed 24 h after injection, and the liver, spleen, kidney, heart, and lung were removed for biochemical and histochemical analyses.

To determine the effectiveness of hGUS and hGUS-GILT at reversing storage pathology, three adult animals in each group were administered three weekly doses (1 mg/kg) of either hGUS, hGUS-GILT, or PBS by injection in the lateral tail vein. Animals were killed 1 week after the third injection, and the organs were removed for histopathology analysis with light or electron microscopy.

Pathology. ***Single-dose study of enzyme distribution.*** For histochemical study, liver, spleen, kidney, intestine, heart, lung, eye, rib and associated marrow, brain, and heart from mice treated with a single dose of either hGUS (n = 5), hGUS-GILT (n = 5), or hGUS-GILT-F1 (n = 4) and killed 24 h later were immersed in Cryo-Gel embedding medium (Instrumedics, Hackensak, NJ) and frozen in liquid nitrogen-cooled isopentane. Sections were stained for GUS activity with the Naphthol-AS-BI histochemical method (38).

Multiple-dose study of response to ERT. Sections (10-μm thick) of liver, kidney, spleen, brain, and adrenal from mice treated with three doses of either hGUS (n = 3), hGUS-GILT (n = 3), or buffer (n = 2) were also stained by using the same histochemical method used for GUS activity. For electron microscopy, liver, spleen, kidney, brain, heart, eye, adrenal, rib, and marrow from the MPS VII mice treated with three doses of hGUS (n = 3), hGUS-GILT (n = 3), or buffer only (n = 2) were collected at necropsy, immersion-fixed in 4% paraformaldehyde/2% glutaraldehyde in PBS, postfixed in osmium tetroxide, and embedded in Spurr's resin. For evaluation of lysosomal storage by light microscopy, toluidine blue-stained 0.5-μm-thick sections were examined. The kidney and rib were also studied by electron microscopy by using a CX100 transmission electron microscope (JEOL) after routine sectioning and staining with uranyl acetate–lead citrate.

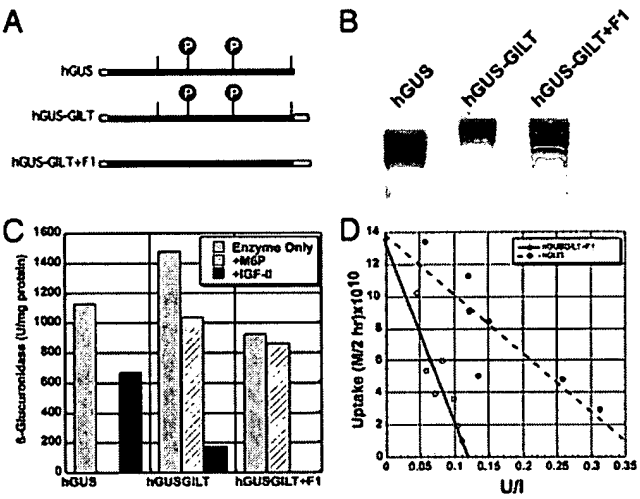

Fig. 1. (*A*) Schematic depiction of the three enzyme preparations used in this study. The position of the four glycosylation sites in native hGUS are indicated by vertical lines. The two glycosylation sites that contain Man6-P (P) are indicated by filled circles. The positions of the signal peptides and GILT tag are indicated by open boxes. hGUS-GILT-F1 is hGUS-GILT that has been treated with the endoglycosidase F1, which removes most of the oligosaccharides, including all of the Man6-P. (*B*) SDS/PAGE of purified recombinant proteins used. (*C*) Uptake of hGUS, hGUS-GILT, and hGUS-GILT-F1 was studied as described in *Methods*. GM4668 cells were incubated with 4,000 units of each enzyme for 3 h in the presence or absence of 2 mM Man6-P (+M6P) or 2.86 mM IGF-II (+IGF-II). Media were removed, the cells were lysed, and GUS activity was determined. Each bar represents a determination from triplicate wells. The observed values of uptake for hGUS plus Man6-P and for hGUS-GILT-F1 plus IGF-II were <1.0 units/mg. (*D*) Determination of K_{uptake}. Enzyme concentrations ranging 1,000–80,000 units/ml were incubated for 2 h in MEM, supplemented with 2 mM L-glutamine and 15% FBS, and processed as described in *Methods* to generate uptake-saturation curves. A double-reciprocal Eadie–Hofstee plot determined the K_{uptake} for each recombinant enzyme. K_{uptake} was determined from titrations of the uptake of untagged hGUS (●) or hGUS-GILT-F1 (○) enzyme. Units on the *x* axis are uptake/input (U/I) or (mol/2)/mol. Units on the *y* axis are mol per 2 × 10^{10}.

Results

GILT Tag-Mediated Uptake of GUS by MPS VII Fibroblasts. Fig. 1*A* is a diagram of the three enzyme preparations used in these studies. Fig. 1*B* shows an SDS/PAGE analysis of the three enzyme preparations. Note the higher M_r of hGUS-GILT, which is because of the presence of the extra 60-aa tag. Also note the reduction in the M_r, which is associated with deglycosylation by treatment of hGUS-GILT with endoglycosidase F1. Fig. 1*C* shows the effect of Man6-P or IGF-II on uptake of the three different enzymes by MPS VII fibroblasts. The uptake of hGUS is inhibited completely by Man6-P, indicating that its uptake relies completely on Man6-P recognition by the IGF-II/CI-MPR. Partial inhibition of hGUS uptake by excess IGF-II is also noted. This finding has been observed (39) and has been attributed to stearic inhibition, rather than competition for the Man6-P binding site. hGUS-GILT shows a different inhibition pattern. Uptake is inhibited only partially by Man6-P and is inhibited more extensively by IGF-II. These observations suggest that its uptake is mediated by both the GILT tag and the Man6-P moieties present on the hGUS-GILT. Finally, endoglycosidase F1-treated hGUS-GILT shows no Man6-P-mediated uptake (no inhibition by Man6-P), but its uptake is inhibited completely by IGF-II (i.e., uptake is completely GILT-mediated). Fig. 1*D* shows a kinetic analysis of the uptake of hGUS and endoglyco-

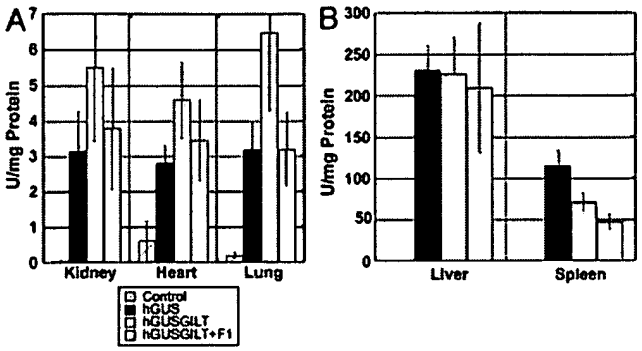

Fig. 2. Biodistribution of hGUS, hGUS-GILT, and hGUS-GILT-F1 after a single injection into MPS VII mice. We infused 1 mg/kg of the indicated enzymes into six MPS VII mice for each treatment (n = 7 for hGUS-GILT) as described in *Methods*. After 24 h, the animals were killed, and tissue samples were processed for biochemistry, as described (11). Levels of enzyme observed in kidney, heart, and lung (*A*) or liver and spleen (*B*) are shown. Crosshatched bars, buffer control cells; black bars, hGUS; gray bars, hGUS-GILT; and white bars, hGUS-GILT-F1. Bars show the average of six to seven animals. Error bars indicate SD. (*A*) Student's t test, indicating that the differences between hGUS and hGUS-GILT observed in kidney, heart, and lung were statistically significant ($P < 0.05$).

sidase F1-treated hGUS-GILT. From these data, we calculate that the K_{uptake} of hGUS is 3.7 nM and that the K_{uptake} of endoglycosidase F1-treated hGUS-GILT, which depends exclusively on GILT-mediated recognition, is 11 nM.

Comparison of the Tissue Distribution of Native and Modified hGUS. We have reported that infused, phosphorylated native mGUS was distributed much more broadly than nonphosphorylated mGUS, which targets nearly exclusively to reticuloendothelial macrophages (11). Fig. 2 compares tissue levels of hGUS in mice 24 h after infusion of buffer only or 1 mg/kg of purified hGUS, hGUS-GILT, or endoglycosidase F1-treated hGUS-GILT. The tissue distribution seen for hGUS is similar to the tissue distribution reported for phosphorylated mGUS (i.e., appreciable delivery to kidney, heart, and lung). hGUS-GILT appeared even more effective at reaching kidney, heart, and lung than native hGUS. Fig. 2 also shows that endoglycosidase F1 deglycosylated hGUS-GILT, which no longer showed any Man6-P-mediated uptake by fibroblasts (Fig. 1*C*), was delivered as effectively to kidney, heart, and lung as the phosphorylated, native hGUS. Fig. 2*B* shows that the liver receives comparable amounts of the three enzymes, although the spleen receives less of the GILT-tagged enzyme and even less of the endoglycosidase F1-treated GILT-tagged enzyme than the native hGUS. This result suggests that removal of the oligosaccharides from the endoglycosidase F1-treated hGUS-GILT diverts much of the enzyme from the reticuloendothelial cells.

Histochemical Analysis of Enzyme Distribution. Fig. 3 *A–C* shows the distribution of each of the three enzymes in liver. In Fig. 3*A*, the hGUS appears to reach both the hepatocytes and reticuloendothelial sinus-lining cells, but the sinus-lining cells stain much more intensely. hGUS-GILT (Fig. 3*B*) and endoglycosidase F1-treated hGUS-GILT (Fig. 3*C*) show a shift in distribution to predominance of staining in the hepatic parenchymal cells. Thus, although there was no quantitative difference in the amount of each enzyme delivered to liver in Fig. 2*B*, there was a qualitative difference in distribution. This result is consistent with the conclusion from the spleen data (Fig. 2*B*) that larger fractions of the GILT-tagged enzymes are diverted from reticuloendothelial cells to parenchymal cells (in this case, from Kupffer cells to hepatocytes).

Fig. 3 *D–F* shows the staining in kidney. In contrast to what was reported for nonphosphorylated mGUS in kidney, which showed no staining (11), all three enzymes showed appreciable staining in kidney, particularly in glomeruli. It is difficult to distinguish between them quantitatively with this technique because of its nonquantitative nature.

Comparison of hGUS and hGUS-GILT in Clearance of Storage in the MPS VII Mouse. To detect quantitative differences in the effectiveness of clearance of lysosomal storage between different forms of the enzyme, we developed a protocol in which enzyme was given over a short course of three weekly treatments with 1 mg/kg enzyme. We examined a wide range of tissues, the same tissues examined in prior studies of ERT in the MPS VII mouse (11, 40–42), 1 week after the last dose. Our initial comparison, which is reported here, was between native hGUS and the GILT-tagged fusion product, hGUS-GILT. The results of this study are summarized in Table 2. Even in this short course of treatment, several tissues were cleared completely (three

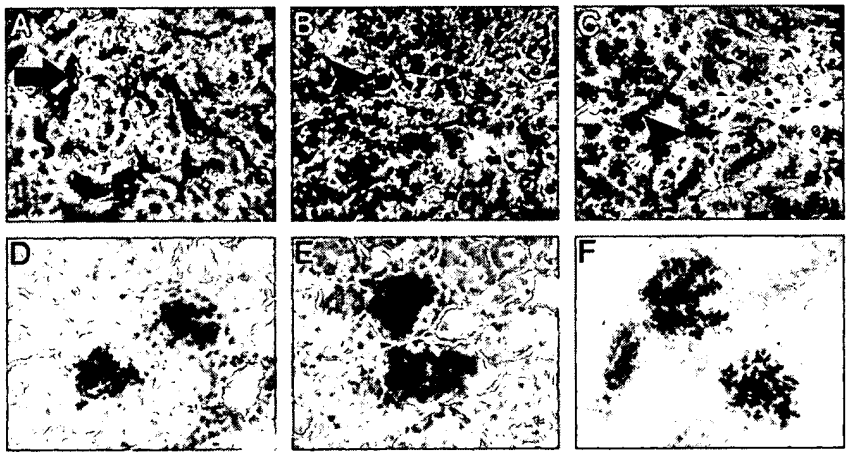

Fig. 3. Histochemical analysis of enzyme localization after a single injection. (*A–C*) After a single infusion of hGUS, enzyme activity was present primarily in the Kupffer cells (arrow), with only a small amount of activity in hepatocytes. Both hepatocytes (arrowheads) and Kupffer cells contain enzyme activity in the hGUS-GILT-treated and hGUS-GILT-F1 mice. (ASBI β-glucuronide, ×400 magnification.) (*D*) Glomeruli from a mouse treated with a single dose of hGUS had activity. (*E* and *F*) After hGUS-GILT and hGUS-GILT-F1 infusion, enzyme was present in the glomeruli in a similar distribution. (ASBI β-glucuronide, ×400 magnification.)

Table 2. Reduction in lysosomal storage after 3 weeks of treatment with two forms of hGUS

Treatment	Kupffer cells	Hepatocytes	Spleen SLC	Renal TE	Glomerular EC	Cornea	RPE	Heart valve	Bone SLC	Bone osteoblasts	Bone marrow	Adrenal IC	Brain neurons/ meninges
hGUS (n = 3)	↓↓↓	↓↓↓	↓↓↓	↓–↓↓	NC	NC	NC	NC–↓	↓↓↓	↓–↓↓	↓↓↓	↓↓↓	NC
hGUS-GILT (n = 3)	↓↓↓	↓↓↓	↓↓↓	↓↓–↓↓↓	↓↓↓	NC	NC	NC–↓↓	↓↓↓	↓↓–↓↓↓	↓↓↓	↓↓↓	NC

SLC, sinus-lining cells; TE, tubular epithelial cells; EC, epithelial cell; RPE, retinal pigment epithelium; IC, interstitial cells; NC, no change (storage similar to untreated mutant). ↓↓↓, marked decrease vacuolization, essentially identical to morphology in the normal animal; ↓↓, moderate decrease in cytoplasmic vacuolization; ↓, slight and/or focal decrease in cytoplasmic vacuolization.

arrows downward) by both enzymes. However, there were two notable differences in which the hGUS-GILT appeared to be more effective in clearing the storage material. First, in kidney, the glomerular visceral epithelial cells and the renal tubular cells had considerably less storage in the hGUS-GILT-treated MPS VII mice. Second, in bone, the osteoblasts were cleared almost completely in the hGUS-GILT-treated mice, whereas the hGUS-treated mice showed minimal or moderate reduction in storage in osteoblasts.

Fig. 4 shows light and electron microscopy of tissues from control, buffer-only-treated MPS VII mice (Fig. 4 *A*, *D*, and *G*), hGUS-treated mice (Fig. 4 *B*, *E*, and *H*), and hGUS-GILT-treated mice (Fig. 4 *C*, *F*, and *I*). The liver sections (Fig. 4 *A–C*) were examined by light microscopy and show essentially complete clearance of storage by both enzymes. The bone sections (Fig. 4 *G–I*), examined by electron microscopy, show clearance of storage in the osteoblasts by hGUS-GILT (Fig. 4*I*), but little difference from control (Fig. 4*G*), in osteoblasts from mice treated with hGUS (Fig. 4*H*). *D–F* show electron microscopic images from glomerular visceral epithelial cells. The hGUS-treated mice (Fig. 4*E*) showed little improvement compared with control (Fig. 4*D*), but the glomerular visceral epithelial cells from the hGUS-GILT-treated mice (Fig. 4*F*) were cleared almost completely.

Discussion

This study had two purposes, namely (*i*) to demonstrate the feasibility of glycosylation-independent enzyme delivery in enzyme deficient fibroblasts and in the mouse model of MPS VII; and (*ii*) to compare the response of MPS VII mice to therapy with tagged or unmodified enzyme. *In vitro* studies of the effect of inhibitors on endocytosis of hGUS-GILT by MPS VII fibroblasts showed clearly that the GILT-tagged enzyme could be taken up by fibroblasts by both Man6-P-mediated and GILT-mediated mechanisms, both of which target the IGF-II/CI-MPR. Treatment of hGUS-GILT with endoglycosidase F1 abolished the Man6-P-dependent uptake but preserved the GILT-mediated uptake.

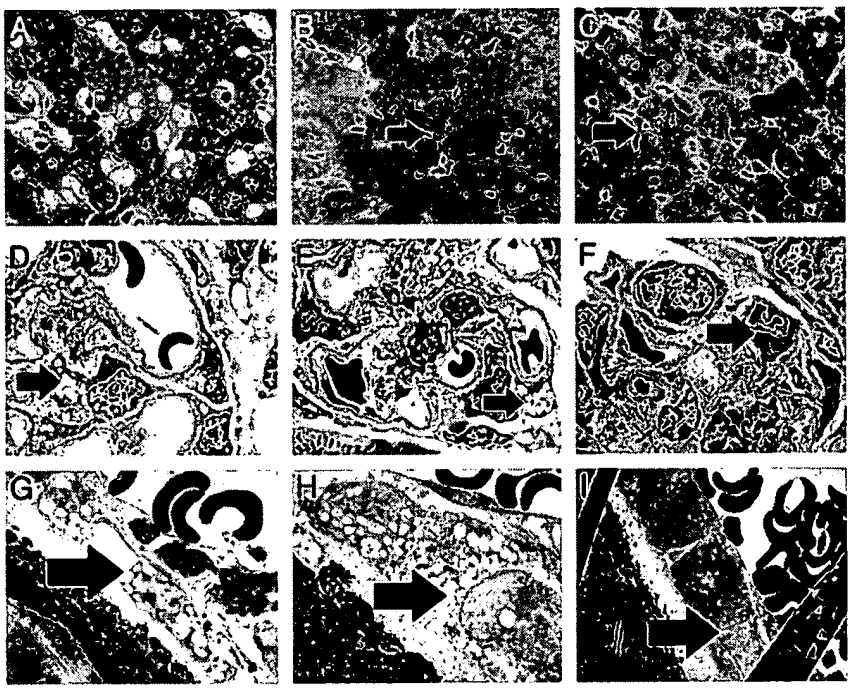

Fig. 4. Reversal of storage after a short course of ERT. (*A*) The liver from an untreated MPS VII mouse had abundant storage in the Kupffer cells (arrow) and a small amount of storage in the hepatocytes. (*B*) After treatment with hGUS, there was a marked reduction in storage in both the hepatocytes and Kupffer cells (arrow). (*C*) A similar reduction in storage in hepatocytes and Kupffer cells (arrow) was present after hGUS-GILT treatment. (*D*) A glomerulus from an untreated MPS VII mouse had abundant lysosomal storage in the visceral epithelial cells (arrow). (*E*) After three injections of hGUS, lysosomal storage in glomerular visceral epithelial cells (arrow) was present in amounts similar to that seen in the untreated MPS VII mouse. (*F*) After treatment with hGUS-GILT, there was a reduction in storage in the glomerular visceral epithelial cells (arrow). (*G*) Osteoblasts (arrow) lining the bone of an untreated MPS VII mouse had lysosomal storage distending the cytoplasm. (*H*) After treatment with hGUS, the osteoblast (arrow) lysosomal storage persisted. (*I*) With hGUS-GILT treatment, the amount of lysosomal storage in osteoblasts (arrow) was markedly reduced. (*A–C*) Toluidine blue. (*D–I*) Uranyl acetate–lead citrate. [Magnifications, ×500 (*A–C*); ×1,428 (*D–F*); ×2,428 (*G*); and ×1,714 (*H–I*).]

The next question addressed was whether infused enzyme could be delivered to physiologically relevant tissues. An earlier study showed the importance of targeting the IGF-II/CI-MPR receptor to reach parenchymal cells in many tissues that do not express the mannose receptor. The studies reported here (Figs. 2 and 3) show that all three forms of hGUS, including the endoglycosidase F1-treated hGUS-GILT, showed the wide distribution seen previously with phosphorylated mGUS but not a preparation lacking Man6-P (11). Furthermore, the enzyme levels in some tissues were higher in the hGUS-GILT-treated mice than in the hGUS-treated mice, suggesting possible value added by the GILT tag.

To compare the effectiveness of GILT-tagged and untagged hGUS at clearing storage from affected tissues in the MPS VII mouse, we used a short course of three weekly injections into adult MPS VII mice, which were examined 1 week after the third injection. The favorable responses for both enzymes were generally similar to those reported previously for the phosphorylated enzyme mGUS. However, the tagged enzyme cleared storage from osteoblasts in bone and visceral glomerular epithelial cells in kidney (podocytes), both sites of storage showing minimal response to untagged enzyme at the same dose.

The enhanced delivery of enzyme to these sites was surprising, given the facts that (*i*) the GILT tag targets the enzymes to the IGF-II/CI-MPR receptor, the same receptor targeted by the Man6-P recognition marker on phosphorylated forms of the enzyme; and (*ii*) the GILT-tagged enzyme appears even less phosphorylated than unmodified hGUS, based on the amount of Man6-P-inhibitable uptake in fibroblasts (Fig. 1*C*). One possible explanation for this finding might be that every monomer on the hGUS-GILT tetramer contains the tag, whereas not even every tetramer of hGUS contains the Man6-P recognition marker. Another possible explanation is that the GILT-tagged enzyme is cleared more slowly after infusion and, therefore, has a greater opportunity to reach cells outside the reticuloendothelial system (liver and spleen), which rapidly removes a larger fraction of the infused, native hGUS.

However, we believe it is unlikely that the GILT tag targets the IGF-I receptor in animals. Because the sites on IGF-II that bind the IGF-II/CI-MPR and IGF-I receptors are distinct (43, 44), it is possible to make mutations in IGF-II that disrupt its binding to the IGF-I receptor and mitogenicity without affecting its affinity for the IGF-II/CI-MPR (42, 45, 46). The GILT tag used in these studies exhibits these desired properties. It contains residues 8–67 of mature human IGF-II fused with a 3-aa bridge to the C terminus of hGUS, lacking the terminal 18 aa. The 8–67 IGF-II reportedly binds to the IGF-I receptor less avidly than native IGF-II (K_d is 30-fold greater than that of IGF-II) but binds more tightly to the CI-MPR (K_d is 11-fold lower) (42). Finally, in uptake experiments with MPS VII fibroblasts, the addition of large molar excesses of IGF-I did not inhibit uptake of hGUS-GILT (data not shown). Thus, the enhanced delivery of hGUS-GILT does not depend on the IGF-I receptor.

Whatever the mechanism, the enhanced delivery of the GILT-tagged enzyme to clinically relevant storage-disease cells makes this approach attractive for study. The GILT-tagged targeting system could be important for replacement of lysosomal enzymes that are poorly phosphorylated in mammalian cells. It could also be helpful in diseases in which enhanced delivery of the missing enzyme to specific cell types, such as osteoblasts or renal glomerular podocytes, could be advantageous.

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